STUDIES OF N-FIXING BACTERIA. VII.

Cytochromes of Azotobacteriaceae.

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Synopsis.

Using a reflectance spectrophotometric method, the representatives of the Azotobacteriaceae were studied. For comparison, three Rhizobia and one other, a Gram-positive bacterium, were also studied and compared.

Azotobacter has the highest QO_2 (4000–5000) (P. W. Wilson, 1951). However, Burke (Wilson, 1951) reported that the effect of pO_2 on *Azotobacter* is independent of the *N* source and cannot indicate the nature of the mechanism of N fixation. Wilson and Fred (Wilson, 1951) and Bond (1950) found that O_2 is connected with the fixation process of N_2 (by legume-plant-*Rhizobium* symbiosis) but direct participation is not necessarily implied. It is thus interesting to investigate the terminal respiratory oxidation enzymes (cytochromes) of N-fixing bacteria. A more detailed study of the cytochromes of *Azotobacter* was carried out by Groucher and Kocholaty (1957), who reviewed the literature of the subject. Using a reflectance spectrophotometer, they reported the absorption spectra of some *Azotobacter*.

The present study compares a wide range of species of *Azotobacter* and *Beijerinckia*. The influence of the presence of NH_4OH which affects the N fixation mechanism (Wilson and Burris, 1947, 1953) is studied in relation to the cytochrome spectra.

Method.

(*a*) *Organisms*: Nearly all known species of *Azotobacter* and *Beijerinckia* were included in the experiment. For comparison, the three groups of *Rhizobium* and *Serratia marcescens* are also included. The classification of Azotobacteriaceae is based on the systematic classification of one of us (Tchan, 1953) combined with Jensen's classification (Jensen, 1955). The cultures were grown on Winogradsky's medium with sucrose, agar, and Mo. For the *Azotomonas insigne*, 2·5% of glucose was added to the above medium. For medium containing ammonium 5 c.c. of 5% ammonium sulphate was used for 100 c.c. of the medium. Cultures were first grown on the N-free medium for 24 hours in a test tube. They were used for inoculation of larger quantities of medium for experimental work. The organisms were harvested in sterile water after 48 to 72 hours of incubation at 25°C. Their purity was checked by microscopical examination.

(*b*) *Reflectance measurements*: The technique used was described by Giovanelli (1947). All the organisms were examined in the reduced state. When oxidized conditions were used, the organisms were washed twice in Winogradsky's medium without sugar and then pure O_2 was bubbled through for several minutes and examined immediately.

Results and Discussion.

(*a*) *General description of cytochrome spectra of Azotobacteriaceae.*

Cytochrome absorption maxima from this and other experiments are shown in the table.

The Sorêt band is present in all organisms examined. Smith reported a band at 4400Å for *Azot. chroococcum*. In the present experiment an absorption band at 4800–4840Å was present in all members of the Azotobacteriaceae. This band was particularly strong for *Az. vinelandii* and *Az. agilis*. However, this band may not be related to the green fluorescent pigment of the above two species since *Beij. derxii*

(Tchan, 1957) has a strong green fluorescence but a weak band at this wave-length. The fluorescent pigments of Azotobacteriaceae have been recently studied by Johnstone (1956). This band seems to be absent (or too weak to be detected) from the *Rhizobium* (peanut). The 5050Å band described by Gourcher and Kocholaty was not found. However, one of us (F.M.) has observed the development of a band in this position from *Aerobacter-aerogenes* when an aerated culture was stored at 4°C. overnight. It is suggested that this band is not sufficiently consistent for the purposes of classification.

TABLE 1.

	Sorêt		flavin		β		αc		$a_1aa_3a_2$	
Azot. chroococcum—										
(Smith)	428	440	..	..	530	..	552	560	590	628
After several transfers:										
(Smith)	427	—	..	..	522	..	552	..	590	..
(Negelein and Gerisher) ..	—	—	..	..	—	..	..	563	—	632
(Goucher and Kocholaty) ..	422	..	..	505	520	..	550	..	590	625
Moss and Tchan	420	480	..	..	524	..	552–4	..	590	632
Azot. vinelandii—										
Wilson and Wilson	420	..	..	..	523	..	551	..	..	625
Fujita and Kodama	..	..	..	..	521	531	550	563	590	632
Goucher and Kocholaty ..	420	..	..	..	525	..	555	..	..	630
Moss and Tchan	418	..	480	..	522	..	552	..	..	632
$+NH_4$	412	..	480	..	525	..	553	..	..	648
Azot. beijerinckii	..	..	..	..	527	..	553	..	..	630
Goucher and Kocholaty ..	422	..	..	..	..	..	..	..	..	..
Moss and Tchan	420	..	480	..	522	..	554	..	589	632
(var. *acidotolerans*)	417	..	480	..	520	..	554	..	589	632
$+NH_4$	416	..	480	..	523	..	553	..	590	633
Azot. macrocytogenes 0—										
Moss and Tchan	422	..	480	..	524	..	557	..	600	632
Azot. macrocytogenes 1—										
Moss and Tchan	423	..	480	..	526	..	558	..	596	632
$+NH_4$	418	..	480	..	524	..	558	..	590	635
Azot. agilis—										
Goucher and Kocholaty ..	427	..	..	505	..	530	..	560	590	625
Moss and Tchan	427	..	480	..	525	..	552	..	590	632
$+NH_4$	420	..	480	..	534	..	..	560	590	636
Azomonas insigne—										
Moss and Tchan	423	..	470	..	522	..	554	..	590	632
$+NH_4$	418	..	480	..	521	..	556	..	590	632
Beij. indica										
Moss and Tchan	418	445	480	..	518	..	551	..	604	
Beij. indica var. *alba*—										
Moss and Tchan	415	..	480	..	518	..	551	..	604	
Beij. lacticogens—										
Moss and Tchan	424	..	480	..	..	525	556	..	598	630
Beij. derxii	418	..	480	..	..	527	555	..	589	630
Rhizobium trifolii—										
Moss and Tchan	422	..	480	..	..	525	555	..	600	640
Rhiz. meliloti—										
Moss and Tchan	418	..	475	..	..	525	555	..	601	..
Rhiz. cow-peat group (Peanut)—										
Moss and Tchan	418	..	..	..	..	523	552	..	604	..
Serratia marcescens—										
Moss and Tchan	422	..	480	..	..	530	..	560	..	632
Bot. beij.	420	..	490	500	..	525	552	..	590	631
mutant white oxidaside	412	..	..	..	..	520	549	..	..	650

Beij. indica and its var. *alba* exhibited a band at 5180Å but not the two other species of the genus (*Beij. derxii* and *Beij. lacticogenes*). No other member of the Azotobacteriaceae or *Rhizobium* possesses this band.

The absorption at 5520–5580Å was obtained with all organisms under examination, but we were unable to observe absorption at 5600–5630Å; *Beij. indica* and its variant *alba* present a band at 5510Å. The absorption band at 5900Å which Goucher and Kocholaty considered as differential of the *chroococcum-agilis* group was observed by us with *Az. beijerinckii* and its variants.

We were able to confirm the presence of a band at 5900Å described by Fujita and Kodama. However, the value of this band for classification of *Azotobacter* has limited value. *Az. macrocytogenes* and *Beijerinckia* and *Rhizobium* present a corresponding absorption band at (5960–6040Å).

The absorption at 6300Å was present in all those examined except the *Rhiz. meliloti*, *Rhizobium* of the cow peat group, *Beij. indica*, and *Beij. indica* var. *alba*.

The *Rhizobium meliloti* has a similar cytochrome spectrum to *Beij. indica* var. *alba*, but the cytochrome spectra of other *Beijerinckia* and *Rhizobium* are different. The *Beij. derxii* and *Beij. lacticogens* have rather similar cytochrome spectra to *Azotobacter*. Between mutants, variants of the same species, some variation of cytochrome spectra was noticed.

Within the same species the recorded cytochrome spectra vary with the authors. It is probable that the organism used and experimental conditions were not comparable.

Concerning the cytochrome spectra of Azotobacteriaceae, it is not possible at present to group the organisms as suggested by Goucher and Kocholaty unless more information is available. Our experiment does not support Derx's hypothesis, which suggests that *Beijerinckia* may be related to the *Rhizobium*.

(*b*) *The influence of Ammonium on the cytochrome spectrum of* Azotobacter.

Wilson (1951) has pointed out that ammonium is completely accepted as a nitrogen source to the exclusion of the nitrogen fixation reaction. It is of particular interest that the addition of NH_4 ion produces *Azotobacter* with a modified cytochrome spectrum. All *Azotobacter* species cultivated in the presence of NH_4 showed a shift of the Sorêt band towards the shorter wave-length. The 6300Å band was generally shifted towards a longer wave-length. There are also modifications of the absorption bands at other wave-lengths but they are not consistent. These modifications become more interesting when the oxidized spectra of cytochromes are compared with those from organisms grown in ammonia. When the organisms were washed and oxygenated, some bands disappeared. A shift of the Sorêt towards the shorter wave-length and 6300Å towards the red was observed. Goucher and Kocholaty made a similar observation.

Nevertheless this aspect of the problem should not be overlooked. If the N fixation is realized by a reduction process of molecular N_2 to NH_3, it is not impossible to imagine that the cytochromes participate in electron transfer of the reduction reactions. Recent work by Hamilton *et al.* (1957) showed that N_2 appears specifically to oxidize flavins and cytochrome b.

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